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Determination of sulphur and nitrogen compounds during the processing of dry fermented sausages and their relation to amino acid generation

Sara Corral[a], Erich Leitner[b], Barbara Siegmund[b], Mónica Flores[a*]

[a]Instituto de Agroquímica y Tecnología de Alimentos (IATA-CSIC) Avda. Agustín Escardino 7, 46980 Paterna, Valencia, Spain

[b] Institute of Analytical Chemistry and Food Chemistry. Graz University of Technology. Stremayrgasse, 9/2, 8010 Graz (Austria)

Corresponding author. Tel.: +34 96 3900022; fax: +34 96 3636301

E-mail address: mflores@iata.csic.es (M. Flores).

Abstract

The identification of odour active sulphur and nitrogen compounds formed during the processing of dry fermented sausages was the objective of this study. In order to elucidate their possible origin, free amino acids (FAAs) were also determined. The volatile compounds present in the dry sausages were extracted using Solvent Assisted Flavour Evaporation (SAFE) and monitored by one and two-dimensional gas chromatography with different detectors; mass spectrometry (MS), nitrogen phosphorous (NPD), flame photometric (FPD) detectors as well as gas chromatography-olfactometry. A total of seventeen sulphur and nitrogen compounds were identified and quantified. Amongst them, 2-acetyl-1-pyrroline was the most potent odour active compound, followed by methional, 2-ethylpyrazine and 2,3-dihydrothiophene characterized by toasted, cooked potato, and nutty notes. The degradation of FAAs, generated during the processing, was related to the production of aroma compounds such as methionine with methional and benzothiazole while ornithine was the precursor compound for 2-acetyl-1-pyrroline and glycine for 2-ethylpyrazine.

Keywords: sulphur, nitrogen, volatile compounds, amino acid, dry fermented sausage.

1. Introduction

The use of one dimensional gas chromatography mass spectrometry (GC-MS) is common to elucidate sausage aroma although the low concentrations of volatile sulphur and nitrogen compounds in meat products, which are in the ppb to ppt range, (Thomas, Mercier, Tournayre, Martin & Berdagué, 2014) require the use of highly sensitive and selective detectors such as flame photometric (FPD), pulsed flame photometric (PFPD), sulphur chemiluminescence (SCD), nitrogen-phosphorus (NPD) or olfactometry (O) detectors. However, the identification of the volatiles can be a laborious task since co-elution of the compounds of interest is common in complex food matrices. A strong tool to achieve a high separation and identification of compounds is two dimensional GC with mass spectrometric detection (heart-cutting 2DGC and comprehensive 2D-GC). Nevertheless, the selection of a suitable technique for the extraction of the volatiles is a crucial point, as the obtained aroma profile is highly dependent on this technique. In this sense, the use of solvent assisted flavour evaporation (SAFE) is regarded to be a good choice to enrich the compounds at trace level such as volatile sulphur and nitrogen compounds (Engel, Bahr & Schieberle, 1999).

Traditional fermented sausages are dried for long ripening periods during which a high number of microbial and chemical reactions take place and the typical sausage flavour is developed (Toldrá, Sanz & Flores, 2001). Fermented sausage flavour is produced by a high number of volatile compounds. Volatile sulphur and nitrogen compounds play a crucial role in the formation of sausage aroma due to their low odour threshold values and their characteristic olfactive notes (Stahnke, 2002). During meat cooking the high temperatures favour the

formation of volatile sulphur and nitrogen compounds (Mottram, 1998) leading to a volatile profile completely different to that reported for the dry-ripening process of meat where low temperatures are applied. Recently, several sulphur containing odorants have been identified in cooked ham and linked to the thermal degradation of thiamine (Thomas et al, 2014). In dry fermented sausages, several odour notes such as onion, roasted nuts, meat broth, rotten egg, cabbage, popcorn, fried snacks or chocolate have been reported, giving rise to the assumption that sulphur compounds play a significant role in flavour formation, even though only a few of them could be identified in previous studies (Corral, Salvador & Flores, 2013; Gianelli, Olivares & Flores, 2011; Olivares, Navarro & Flores, 2011). In addition, it was revealed that these notes were crucial to achieve the aroma of fermented sausages in aroma reformulation studies (Corral, Salvador & Flores, 2015; Söllner & Schieberle, 2009). Therefore, the further identification of these compounds has remained a challenge for the dry fermented sausage industry to determine the mechanism related to their generation and their effect on the final sensory quality of the products.

Several mechanisms are involved in the generation of sulphur compounds of dry fermented sausages. However, it is well known that sulphur containing amino acids (cysteine and methionine) together with thiamine, are precursors of these volatile sulphur compounds (Mottram, 1998). Pork contains the highest thiamine content of any meat type (Mottram, 1991), but the generation of large amounts of free amino acids produced during dry fermented sausage due to endogenous proteolytic activities (Toldrá, et al., 2001) may act as essential source of volatile compounds. The degradation of methionine and

cysteine leads to the formation of methanethiol and hydrogen sulfide, respectively, which - owing to their high reactivity - generate other volatile sulphur compounds (Mottram, 1991). On the other hand, volatile nitrogen compounds can be formed via Strecker degradation from a nitrogen source such as any amino acid produced (Meynier & Mottram, 1995). Whilst enzymatic endogenous activity is predominant at the beginning of the ripening process, the microorganisms' metabolisms play a crucial role during the amino acid degradation to volatile compounds (Molly, Demeyer, Raemaekers, Ghistelinck & Geenen, 1997). Thus, the relationship between the generation of free amino acids and volatile sulphur and nitrogen compounds can be useful to understand the mechanism of production of these compounds in dry fermented sausages.

Therefore, the aim of the present work was to identify the odour active volatile sulphur and nitrogen compounds produced during the ripening of dry fermented sausages. Furthermore, the generation of free amino acids was followed during processing in order to elucidate their contribution to the production of sulphur and nitrogen odour active compounds.

2. Material and methods

2.1. Dry fermented sausage processing

Dry fermented sausages were manufactured using lean pork (75 %), pork back fat (25 %) as well as the subsequently mentioned additives added in g/Kg to the sausage formulation: lactose (20); dextrin (20); sodium caseinate (20); glucose (7); sodium ascorbate (0.5); sodium nitrite (0.15); potassium nitrate (0.15) and starter culture (0.1) C-P-77-S bactoferm (Chr. Inc. Hansen, Denmark) containing *Lactobacillus pentosus and Staphylococcus carnosus*.

The manufacturing process was described by Corral et al. (2013). The meat mixture was stuffed into collagen casings of 9.5 cm diameter (FIBRAN, S.A., Girona, Spain) and the sausages were subjected to drying at an initial stage of 15-20 ºC and 75-85 % relative humidity (HR) for 24 h, followed by ripening at 9 ºC and 75-85 % HR for 66 days. In order to control the ripening process, weight losses and pH were measured during processing (Corral et al., 2013).

Three sausages were randomly collected at 0, 15, 36 and 66 days to study the effect of ripening time on the formation of volatile sulphur and nitrogen compounds. Several sausage slices (1 cm thickness) were wrapped in aluminium foil, vacuum packaged and stored at -80 ºC for the analysis of the volatile compounds and free amino acids. In addition, the moisture of the sausage and the water activity were measured from the minced sausages as described by Corral et al. (2013). All results were expressed as the mean of three replicates at each sampling time.

2.2. Isolation of volatiles by solvent assisted flavour evaporation (SAFE)

Dry fermented sausages (100 g) were cut into pieces, frozen with liquid nitrogen and powdered in a blender (Waring Laboratory, USA). 2-Methyl-3-heptanone (5.1 µg) (Sigma-Aldrich, Germany) was used as internal standard and added directly to the sausage powder. The powder was extracted three times with dichloromethane (1:2, w:v) (total volume 600 ml). After drying over anhydrous sodium sulphate, the volatiles were isolated using the solvent-assisted flavour evaporation (SAFE) technique (Engel et al, 1999). The obtained extract was dried over anhydrous sodium sulphate and concentrated at 45 ºC to a final volume of 500 µl by distilling off the solvent by means of a

Vigreux column under a stream of nitrogen. This procedure was performed in triplicate at each sampling time.

2.3. Analysis of volatile sulphur and nitrogen compounds

After the isolation of sulphur and nitrogen compounds from the fermented sausages, the SAFE extract was analysed by GC and multidimensional GC (MDGC) in combination with different detection systems (mass selective detector, flame photometric and nitrogen-phosphorous detectors). Different GC equipments were used and all of them were equipped with an autosampler CTC Combi Pal (CTC Analytics AG, Zwingen, Switzerland).

2.3.1. Separation of volatiles by GC-MS analysis

An Agilent 7890 GC coupled with a 5975 MS detector (Hewlett Packard, Palo Alto, CA) was used. The SAFE extract was injected into the injection port at 240 ºC using split injection (split ratio 10:1). For the separation of the volatiles, two different columns were used; a capillary column HP-5 (30 m length, 0.25 mm i.d., 1 µm film thickness) (Agilent Technologies, USA) used with helium as carrier gas at a 33 cm/sec. The GC oven temperature was -10 ºC for 1 min (cooling with liquid nitrogen), ramped to 240 ºC at 8 ºC/min and then to 280 ºC at 40 ºC/min. The MS interface temperature was set to 280 ºC. The second column used was a capillary column DB-624 (30 m length, 0.25 mm i.d., 1.4 µm film thickness) used with helium as carrier gas at a 34 cm/sec. The GC oven temperature was 38 ºC for 13 min, ramped to 100 ºC at 3 ºC/min and held at 100 ºC for 5 min, then to 150 ºC at 4 ºC/min and to 210 ºC at 5 ºC/min and finally, held at 210 ºC for 10 min. The MS interface temperature was set to 240 ºC. Mass spectra were obtained by electron impact ionisation at 70 eV, and data were acquired across the range 29-400 amu. Linear retention indices (LRI)

of the volatile compounds were calculated using the series of n-alkanes (Aldrich, Germany) for both columns. Compounds were identified by comparison of the obtained mass spectra with mass spectra from the library databases (Nist'05 and '08), as well as by comparing the calculated linear retention indices (Kovats, 1965) with those from literature (http://www.flavornet.org/flavornet.html or http://webbook.nist.gov/chemistry) as well as from authentic reference compounds (thiazole, 2,4-dimethylthiazole, 2-acethylthiazole, benzothiazole, 3-methylthiophene, 2-methylpyrazine, 2-ethylpyrazine, methional, methionol, 2-ethylpyridine, dimethylsulfone (Sigma-Aldrich, Germany); 2-acetyl-1-pyrroline; 2-acetylpyrrole, 2,6-dimethylpyrazine, 2-methyl-3-furanthiol (Safc, USA); pyrrole (Fluka, Germany) and 2-acetyl-1-pyrroline (synthesised according to Deblander et al., 2015).

2.3.2. Separation of sulphur volatiles by GC-FID-FPD

Volatile sulphur compounds in the SAFE extract were detected using a flame photometric detector (FPD) installed on an Agilent 6890 GC that was equipped with a simultaneously operating FID detector (Hewlett Packard, Palo Alto, CA). The SAFE extract was injected into the injection port at 220 ºC (split injection; split ratio 15:1) and helium was used as carrier gas at a constant flow rate of 26.3 cm/sec. The compounds were separated on a capillary column ZB-5 (30 m length, 0.25 mm i.d., 1 μm film thickness) (Phenomenex, Inc). The GC oven temperature was -10 ºC for 1 min (cooling the oven with liquid nitrogen), ramped to 280 ºC at 12 ºC/min and kept at 280 ºC for 3 minutes. FID and FPD temperatures were 280 ºC and 240 ºC, respectively. Linear retention indices of the eluted volatile compounds were calculated as indicated above.

2.3.3. Separation of sulphur volatiles by MDGC

The MDGC system from Shimadzu (Japan) QP-2010 consisted of two independent gas chromatographs interconnected by means of a Deans switch device (Valco Instruments, Houston, TX, USA). Chromatograph 1 (GC1) was equipped with a SPL-2010 (Plus) injection port, a Deans switching device and an FID detector. The SAFE extract was injected at 220 ºC (split injection; split ratio 5:1) and helium gas as carrier gas at a linear velocity of 16.8 cm/sec. The compounds were separated on a capillary column Optima 5 Accent (30 m length, 0.25 mm i.d., 0.25 µm film thickness) (Macherey-Nagel GmbH & Co. KG, Germany). The GC oven temperature was -10 ºC for 1 min (cooling with liquid nitrogen), ramped to 260 ºC at 7 ºC/min and held at 280 ºC for 7 min. The FID temperature was kept at 300 ºC. Chromatograph 2 (GC2) was coupled to an MS detector. The column was connected to the Deans switch placed in the first chromatograph via the thermostated transfer line held at 250ºC. The compounds were separated on a fused silica capillary column ZB-WAX plus (30 m length, 0.25 mm i.d., 0.25 µm thickness). The oven temperature program was 40 ºC for 1 min, ramped to 240 ºC at 6ºC/min and held at 240 ºC for 2 min. The ion source and interface temperature were 200 and 220 ºC, respectively. Mass spectra were obtained by electron impact ionisation operated at 70 eV, and data were acquired across the range 46-200 amu in the SCAN mode as well as in the SIM mode using the following specific m/z ratios of ions 97 and 98 for 3-methylthiophene, 76, 80, 104, 122 for methional and 61 and 106 for methionol. The identification of sulphur compounds was performed by comparing the mass spectra to those from the library database (NIST) and confirmed by injection of the pure reference compounds analysed under the same experimental conditions.

2.3.4. Separation of nitrogen volatiles by GC-NPD-PID

Volatile nitrogen compounds from SAFE extract were detected using a nitrogen-phosphorous detector (NPD) installed on an Agilent 7890 GC also equipped with a photo ionization detector (PID) (SRI Instruments, California, USA). The SAFE extract was injected into the injection port at 250 ºC (split injection; split ratio 8:1) and helium was used as carrier gas at a flow rate of 17.69 cm/sec. The compounds were separated on a fused silica capillary column ZB-5 (30 m length, 0.25 mm i.d., 1 μm film thickness) (Phenomenex, Inc). The GC oven temperature was -10 ºC for 1 min (cooling the oven with liquid nitrogen), ramped to 240 ºC at 8 ºC/min and then to 280 ºC at 40 ºC/min. NPD and PID temperature was 200 ºC. Auxiliary temperature was 280 ºC. Linear retention indices of the volatile compounds were calculated as indicated above.

2.3.5. Quantification of volatile sulphur and nitrogen compounds

The relative quantification of the identified volatile compounds was performed as described in the GC-MS section (see 2.3.1.). For most compounds, the quantification was performed in the SCAN mode using the extracted ion chromatogram (TIC or EIC) with the exception of 2-acetyl-1-pyrroline and 2-methyl-3-furanthiol which were relatively quantified in the SIM mode using the mass-to-charge ratio (m/z) area of the characteristic ions 43 and 111, respectively. The volatile compounds were relatively quantified by comparison to the internal standard (2-methyl-3-heptanone) and expressed as ng/g dry matter.

2.4. Sensory evaluation of sulphur and nitrogen compounds by gas chromatography olfactometry (GC-O)

The SAFE extracts were subjected to GC-olfactometry using a gas chromatograph (Agilent 6890, USA) equipped with a flame ionization detector (FID) and a sniffing port (ODP3, Gerstel, Mühlheim an der Ruhr, Germany) and a capillary column DB-624 (60 m, 0.32 mm i.d., film thickness 1.8 µm) (Olivares et al., 2011). The aroma impact of volatile compounds was determined by means of aroma extract dilution analysis (AEDA) (Ulrich & Grosch, 1987) by dilution of the SAFE extract with dichloromethane. One microliter of each dilution was injected into the GC and analysed by four experienced assessors until no odours were detected. Flavour dilution factor (FD factor) was assigned to the highest dilution at which an odour active compound was detected. Aroma compounds were identified using the following techniques: comparison with mass spectra, comparison with LRI of authentic standards injected in the GC-MS and GC-FID-O, and by coincidence of the assessors's descriptors with those described in the Fenaroli's handbook of flavour ingredients (Burdock, 2002).

2.5. Analysis of free amino acids

The content of free amino acids in the fermented sausages was determined according to Aristoy & Toldrá (1991) using norleucine (65.6 µg) as internal standard. Phenylthiocarbamyl amino acids derivatives were analysed by reversed-phase HPLC (1200 Series Agilent chromatograph; Agilent, Palo Alto, CA, USA) using a Waters Nova Pack C18 column (3.9 x 300mm) (Waters Corporation, Milford, USA) and ultraviolet detection (254nm) as described by Flores, Aristoy, Spanier & Toldrá (1997). The concentration of reduced glutathione, cysteine, glutathione and cystine was determined as described by Marušić, Aristoy & Toldrá (2013) using an Agilent 1100 Series HPLC with

fluorescence detection (λ_{ex} = 330 nm, λ_{em} = 376 nm). The analysis was performed in three sausages at each sampling time and each sausage sample was derivatized in triplicate. The results were expressed as mg/100 g sausage in dry matter.

2.6. Statistical analysis.

The effect of ripening time on the sulphur and nitrogen compounds and free amino acids content was performed by one factor ANOVA analysis using the statistic software XLSTAT (v 2009.4.03, Addinsoft, Barcelona, Spain). Fisher's test was used to evaluate differences among ripening times. A Pearson correlation procedure was performed to evaluate any relationship between the concentrations of sulphur and nitrogen containing volatiles and the free amino acids.

3. Results and discussion

The use of conventional 1-D GC-MS allowed the identification of only four volatile sulphur and nitrogen compounds in the SAFE extract of dry fermented sausages. However, the use of specific detectors, FPD and NPD, and two dimensional GC-MS increased the sensitivity to volatile sulphur and nitrogen compounds. Figure 1A gives an example of chromatograms showing the traces obtained by FPD and MDGC of a SAFE extract of the sausage. Three signals were selected at LRI (HP-5) of 776, 908 and 981 LRI according to FPD signal. Therefore, three heart-cuts were transferred onto a second column (Fig. 1B). The corresponding chromatograms obtained from the second dimension are shown in Fig. 1C, 1D, 1E. The sulphur compounds identified in each heart-cut were 3-methylthiophene, methional and methionol, respectively.

On the other hand, the identification of volatile nitrogen compounds was performed using an NPD detector and 1-D GC-MS leading to the identification of ten volatile nitrogen compounds (Table 1).

A total of seventeen sulphur and nitrogen compounds were identified and confirmed with authentic standards except 2,3-dihydrothiophene which was identified according to its mass spectra by Nist'05 (Table 1 and Fig. 1). The identified volatile compounds were relatively quantified in course of the sausage ripening process at 0, 15, 36 and 66 days (Table 1). Different chemical structures were identified: thiazoles (4), pyrroles (3), thiophenes (2), pyrazines (3), methionine derived compounds (2), thiols (1), pyridines (1), and others (1). Thiazole, 2,4-dimethylthiazole, 2-acethylthiazole, 2-ethylpyrazine and 2-ethylpyridine have not been reported previously in dry fermented sausages (Corral et al., 2013; Gianelli et al., 2011; Söllner & Schieberle, 2009). In general, all sulphur and nitrogen compounds showed an increase in concentration throughout the ripening process. At the same time, the measurement of free amino acids in the analysed sausages also showed an increase in concentration (Table 2). However, the highest increase was observed during the first stages of processing until 36 days, even though few of them (Thr, Ile, Lys, Asn) increased in concentration until 66 days. The low increase in course of the last ripening stages indicates a further degradation of the released amino acids by the microbial fermentation activity present in the sausages to produce volatile compounds (Ravyts, Vuyst & Leroy, 2012).

Due to the low threshold of volatile sulphur and nitrogen compounds (Stahnke, 2002), GC-olfactometry was performed revealing nine aroma active compounds of the seventeen compounds identified (Table 1). Pyrrole, 2-

ethylpyridine and 2-ethylpyrazine, have not been reported before as aroma active compounds in dry fermented sausages (Corral et al., 2013, 2015; Gianelli et al., 2011; Olivares et al., 2011; Söllner & Schierberle, 2009; Schmidt & Berger, 1998). On the other hand, 3-methylthiophene and 2-methyl-3-furanthiol, previously reported as aroma active compounds in dry fermented sausages (Corral et al., 2013; Söllner & Schieberle, 2009), did not show influence on the aroma indicating that in these products their concentrations were below their odour thresholds. In our experiments, 2-acetyl-1-pyrroline was the most potent odour active compound followed by methional, 2-ethylpyrazine and 2,3-dihydrothiophene and followed by pyrrole, 2-ethylpyridine, 2,6-dimethylpyrazine, 2-acetylpyrrole and benzothiazole (Table 1).

Regarding the generation of odour active compounds in the course of dry sausage processing, benzothiazole concentration reached its maximum at 36 days of process. Thiazole compounds are mainly formed by two pathways; thiamine acid degradation or by non-enzymatic browning reactions between reducing sugars and amino acids in the presence of hydrogen sulphide (H_2S) originated from the degradation of sulphur containing amino acids (Güntert et al., 1990). In cooked ham, several thiazole compounds have been found, probably as result of the thermal degradation of thiamine (Thomas et al., 2014). However, the ripening process in dry fermented sausages does not reach high temperatures to favour the generation of these thiazole compounds even if the generation of 2-acetylthiazole had been reported before in model systems under mild conditions, low temperatures and low pH values (Pripis-Nicolau, De Revel, Bertrand & Maujean (2000). Concerning their relation to the concentration of the corresponding amino acids , the high increase of

methionine and in low proportion of cysteine (Table 2) may favour their generation. In this sense, a positive correlation between benzothiazole with the amino acid methionine (Table 1, supplementary material) was observed. This finding confirmed the origin from sulphur containing amino acid as reported by Meynier & Mottram (1995) who detected thiazole and 2-acetylthiazole in heated model systems containing cysteine and ribose and favoured at a pH range of 5.5 to 6.5.

Regarding odour active pyrrol compounds, pyrrole, 2-acetyl-1-pyrroline and 2-acetylpyrrole were relatively quantified. Their relative concentration increased throughout the process being significantly higher at the end of the process for pyrrole and 2-acetyl-1-pyrroline. These compounds have been previously described as being derived from amino acid pyrolysis (proline), reaction of ammonia with dicarbonyls derived from the breakdown of Amadori products or interaction of furfurals and ammonia (Mottram, 1991). In the dry fermented process, they can be generated from microbial amino acid degradation (Stahnke, 2002). 2-Acetyl-1-pyrroline is a potent odorant in meat products (Söllner & Schieberle, 2009) which has been mainly found in Mediterranean sausages covered with yeast (Demeyer et al., 2000) and its formation was attributed to yeast degradation of proline and ornithine amino acids (Schieberle, 1990). The results of our studies confirmed these findings, as a positive correlation between 2-acetyl-1-pyrroline concentration and ornithine (Table 1, supplementary material) was obtained in the dry fermented sausages.

Concerning odour active thiophene compounds, 2,3-dihydrothiophene concentration significantly increased in course of the fermentation until 66 days. In dry fermented sausages, thiophenes can be originated by yeast metabolism

such as from *Debaryomyces hansenii*; though their production depends on its metabolic activity which is strain dependent (López Del Castillo-Lozano, Delile, Spinnler, Bonnarme & Landaud, 2007). However, their formation have also been reported by means of the reaction of hydrogen sulfide or other volatile sulphur compounds derived from sulphur containing amino acids, with sugar degradation products from Maillard reaction, the heating of furans with hydrogen sulfide or the thermal degradation of thiamine (Mottram, 1991).

With regard to pyrazines, 2,6-dimethylpyrazine and 2-ethylpyrazine actively contributed to sausage aroma. 2-Ethylpyrazine significantly increased in course of the process while the 2,6-dimethylpyrazine content decreased after 15 days. Generally, alkyl pyrazines are formed from a carbohydrate/amine system where the carbohydrates act as the carbon source and amino acids act as the nitrogen source. As a consequence, all amino acids can act as precursor compounds. The results obtained showed a positive correlation for 2-ethylpyrazine with glycine (Table 1, supplementary material) (Meynier & Mottram, 1995). Pyrazine formation is favoured by high temperatures, basic pH, carnosine presence or low moisture (Fig. 2) (Jayasena, Ahn, Nam & Jo, 2013).

Regarding compounds that were derived from methionine, , only methional contributed to sausage aroma. Methionine derived compounds can be formed by Strecker degradation or microbial enzyme activity (Martínez-Cuesta, Peláez & Requena, 2013). The conditions in the dry sausage, as well as during the fermentation process, favour the microbial activity (lactic acid bacteria and yeast) to produce them. During the sausage processing, methional concentration significantly increased at 36 days continuing to increase until 66

days. A positive correlation was observed for methional and methionine (Table 1, supplementary material).

In relation to odour active pyridines, 2-ethylpyridine concentration did not show significant changes throughout the fermentation process. 2-Ethylpyridine could have been formed from aspartic acid and isoleucine amino acids (Hwang, Hartman & Ho, 1995) and 2,4-heptadienal (E,E) (Elmore, Campo, Enser & Mottram, 2002).

In summary, several positive correlations were found between aroma active compounds and amino acids. Different factors can be involved in the formation of these compounds during the dry fermentation of sausages. The main pathways for their formation are chemical, such as Maillard or microbial reactions. In general, the Maillard reaction requires high temperatures which do not take place in dry fermented sausage processing. However, this study clearly demonstrated how the long ripening time and the presence of high amounts of free amino acids, obviously favour these reactions even at the low temperatures of the fermentation process (Ventanas, Estévez, Delgado & Ruiz, 2007). As observed in our study, volatile sulphur compounds arose mainly from methionine as it was present at higher concentrations than cysteine, cystine (cys-cys) and tripeptide GSH (Glu-Cys-Gly) (Marušić et al., 2013). However, the hydrolysis of GSH and cystine may be a source of the constituents' amino acids and therefore, cysteine will be available to be microbially metabolized during sausage processing. This microbial activity has been confirmed previously by a high abundance of volatile sulphur compounds in dry sausages inoculated with *Staphylococcus carnosus* (Tjener, Stahnke, Andersen & Martinussen 2004) and *D. hansenii* strains (Cano-García, Rivera-Jiménez, Belloch & Flores, 2014).

Nevertheless, scarce microbial mechanisms have been proposed for the generation of nitrogen containing compounds like pyrazines and pyridines (Larroche, Besson & Gros, 1999; Cheng, Reineccius, Bjorklund & Leete, 1991). However, one study performed in a model system under mild conditions using low temperatures and low pH indicated the formation of these compounds especially in the presence of cysteine (Pripis-Nicolau et al., 2000). Nevertheless, the addition of sodium nitrite and potassium nitrate to sausage formulation could act as source of volatile nitrogen compounds. As a result of their addition to the dry fermented sausage, several nitriles, nitro-alkanes or nitrates were reported previously (Stahnke, 1995, 2002). Nevertheless, such compounds could not be detected in our study. In contrast, Thomas et al. (2013) reported that nitrite is not directly involved in the production of odorous compounds but in its absence the fatty acid oxidation is favoured masking the odour of sulphur compounds responsible for the typical aroma of nitrite cured meat products. For that reason, further studies should be carried out to control the processing factors that affect sulphur and nitrogen compounds generation and, as a consequence, the final sausage aroma.

4. Conclusion

The use of specific detectors (NPD and FPD) and two dimensional GC-MS revealed the generation of volatile sulphur and nitrogen compounds throughout the ripening process of dry sausages. Among the seventeen volatile compounds relatively quantified, 2-acetyl-1-pyrroline was the most potent odour active compound, followed by methional, 2-ethylpyrazine and 2,3-dihydrothiophene characterized by toasted, cooked potato, and nutty notes. The

sausage environment, acid pH, low a_w, high concentration of free amino acids and especially methionine, cysteine and, cystine and GSH as a source of cysteine, favoured the formation of sulphur compounds (methional and benzothiazole). In addition, the microbial degradation of ornithine produced 2-acetyl-1-pyrroline while glycine degradation generated 2-ethylpyrazine.

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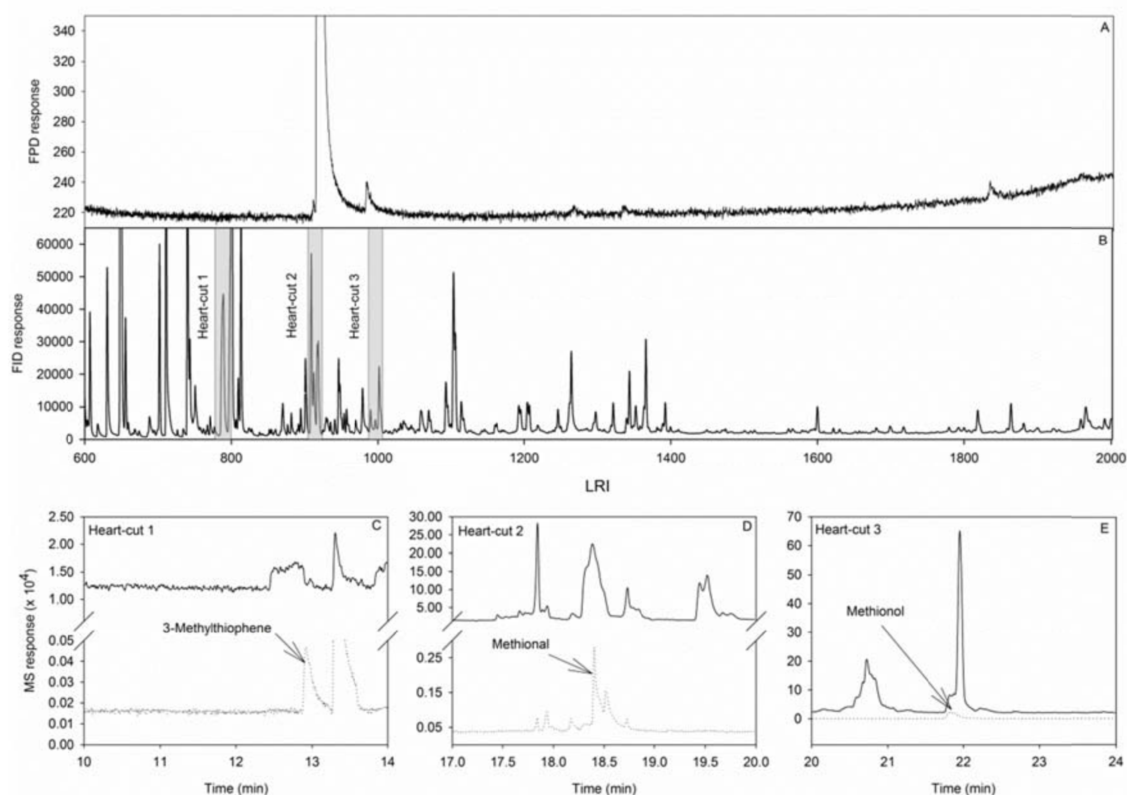
Figure legends

Fig. 1. Sulphur compounds detected by FPD and separated by MDGC (heart-cut technique) in dry fermented sausages at the end of process (66 days). A) FPD chromatogram, B) First dimension chromatogram of MDGC system. C,D,E) Second dimension chromatogram of each heart-cut shown in graph B: dotted line (SIM response) and straight line (SCAN response) for each compound.



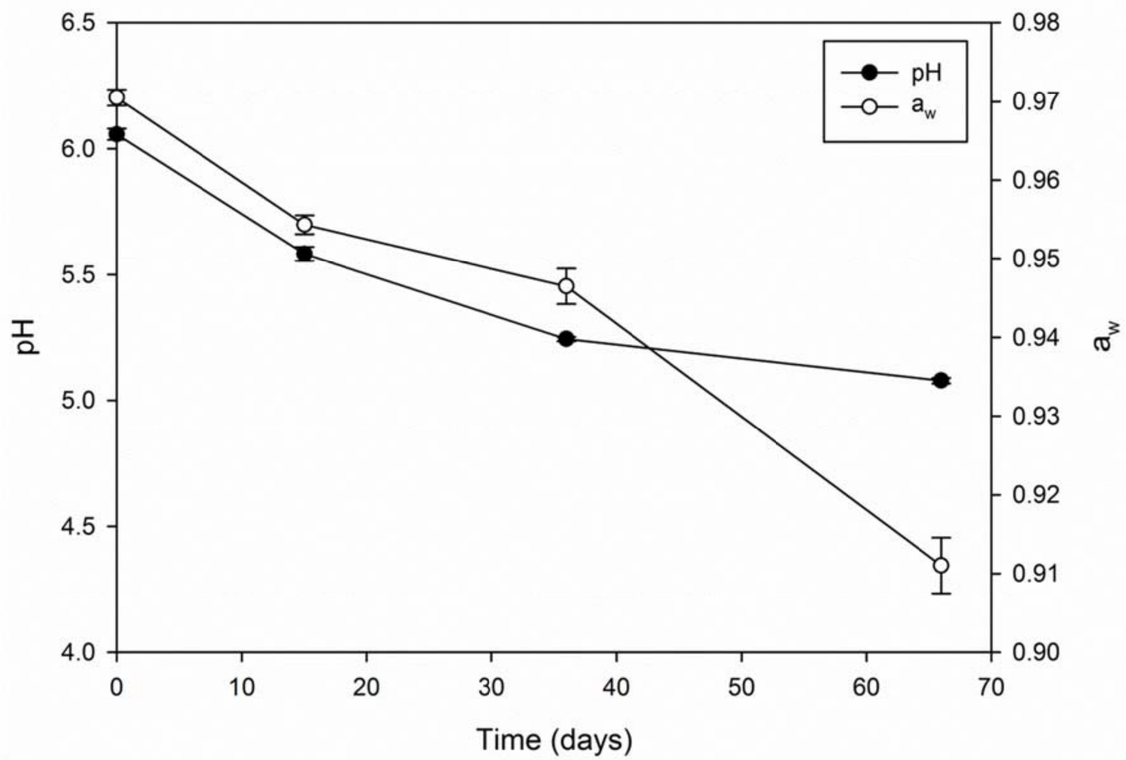


Fig. 2. Evolution of pH and aw throughout the ripening of dry fermented sausages.

Table 1. Sulphur and nitrogen compounds generated during the ripening of dry fermented sausages.

	HP-5		DB-624		Time (days)											
Compound	**LRI[a] comp**	**LRI[b] std**	**LRI[a] comp**	**LRI[b] std**	**0**		**15**		**36**		**66**		**SEM[c]**	**LRI[d] GC-O**	**Descriptor**	**FD[e] factor**
Thiazoles					**19.23[f]**	**c**	**41.64**	**b**	**52.23**	**a**	**51.17**	**a**	**4.17**			
Thiazole	745	742	776	776	1.94	b	10.90	a	11.65	a	18.11	a	2.18			
2,4-Dimethylthiazole	894	892	910	913			0.27		0.58		1.50		0.53			
2-Acetylthiazole	1018	1020	1074	1076			0.63	b	1.65	a			0.11			
Benzothiazole	1233	1232	1292	1292	17.29	b	29.84	ab	38.36	a	31.55	a	4.36	1298	Green, strange, damp, acid	1
Pyrroles					**29.29**	**c**	**69.20**	**bc**	**147.40**	**b**	**279.37**	**a**	**26.76**			
Pyrrole	767	762	840	842			1.26	b	2.69	b	17.71	a	1.53	848	Coffe, sweet	1
2-Acetyl-1-pyrroline	930	931	958	960	28.83	c	66.06	bc	142.71	b	260.05	a	26.40	962	Fried corn, toasted, roasted meat, crust	50
2-Acetylpyrrole	1060	1060	1158	1155	0.46		1.88		2.00		1.62		0.65	1149	Toasted, strong, bitter	1
Thiophenes					**34.88**	**b**	**34.95**	**b**	**60.00**	**b**	**135.58**	**a**	**11.05**			
3-Methylthiophene	776	776		803							1.92		0.50			
2,3-Dihydrothiophene[g]	1219	na[h]	1388	na	34.88	b	34.95	b	60.00	b	133.65	a	11.08	1386	Walnut or hazelnut, acid	2
Pyrazines					**7.83**	**ab**	**3.32**	**c**	**5.71**	**b**	**10.50**	**a**	**0.91**			
2-Methylpyrazine	839	839	865	860	1.52		2.25		2.75		2.60		0.68			
2,6-Dimethylpyrazine	919	914	943	943	5.57	a	0.53	b	1.25	b	0.92	b	0.64	945	Toasted, spicy, acid	1
2-Ethylpyrazine	918	918	947	946	0.74	b	0.53	b	1.70	b	6.98	a	0.30	949	Toasted, spicy, acid	2
Methionine derived					**1.02**	**c**	**56.66**	**b**	**109.74**	**a**	**13.79**	**c**	**10.97**			
Methional	908	908	964	964	0.72	b	3.49	ab	5.57	a	6.70	a	1.23	968	Cooked potate, cauliflower	10
Methionol	981	981	1062	1062	0.30	c	53.17	b	104.17	a	7.09	c	9.59			
Thiols																
2-Methyl-3-furanthiol	868	868	888	894	31.95		37.98		34.75		24.91		12.98			
Pyridines																
2-Ethylpyridine	908	906	935	934	0.54		0.83		1.07		0.60		0.15	936	Rotten mushroom, detergent	1
Others																
Dimethyl-sulfone	923	925	1060	1060	1769.35		1287.34		1512.44		1503.86		606.00			

[a] Linear retention indices (LRI) of the compounds eluted from the GC-MS using a HP-5 capillary column (J&W Scientific 30 m x 0.25 mm i.d. x 0.25 µm film thickness) or a DB-624 capillary column (J&W Scientific 30 m x 0.25 mm i.d. x 1.4 µm film thickness).

[b] Linear retention indices from pure standard or database (http://www.flavornet.org/flavornet.html or http://webbook.nist.gov/chemistry)

[c] Standard error of the mean.

[d] Linear retention indices (LRI) of the compounds or standards eluted from the GC-FID-O using a DB-624 capillary column (J&W Scientific 60 m x 0.32 mm i.d. x 1.8 µm film thickness).

[e] FD Flavor dilution factor.

[f] Estimated quantities in ng/g of sausage calculated by comparison with IS (2-methyl-3-heptanone); Different letters in each row indicate significant differences at $p<0.05$ (Fisher's test).

[g] Tentative identification by mass spectrum.

[h] No available

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4 **Table 2 .** Concentration of free amino acids during the ripening of dry fermented
5 sausages (mg/100 g dry matter).

Amino acid	Time (days) 0		15		36		66		SEM[a]
Essential									
His	6.89	c[b]	25.19	b	49.70	a	59.19	a	4.89
Thr	11.37	d	28.96	c	63.88	b	84.58	a	5.11
Val	15.02	c	65.74	b	131.17	a	138.52	a	5.26
Met	5.01	d	25.83	c	61.77	a	55.79	b	1.36
Ile	10.43	d	37.55	c	82.66	b	95.45	a	1.90
Leu	14.89	c	141.14	b	275.73	a	271.52	a	3.85
Phe	9.56	c	64.12	b	129.05	a	128.97	a	2.82
Trp	3.35	d	16.70	c	30.70	a	26.20	b	0.84
Lys	17.06	c	62.96	c	141.00	b	185.50	a	15.39
Nonessential									
Asp	41.67	d	99.17	c	193.88	a	153.83	b	9.46
Glu	26.32	c	77.87	b	135.33	a	112.65	a	8.10
Ser	11.81	c	35.71	b	58.12	a	29.57	bc	6.99
Asn	3.50	d	13.89	c	26.79	b	33.44	a	2.04
Gly	27.35	b	35.41	b	60.12	a	65.50	a	4.02
Gln	91.17		99.89		117.73		100.00		10.67
Ala	74.77	b	108.69	b	166.77	a	172.42	a	11.57
Arg	20.76	a	20.96	a	9.61	c	14.56	b	1.37
Pro	10.65	c	86.46	b	151.82	a	137.16	a	5.84
Tyr	11.17	c	40.29	b	53.05	a	41.46	b	2.43
Orn	0.58	c	18.18	b	41.99	a	47.66	a	4.78
Cys	10.08	c	14.32	bc	18.90	a	17.11	ab	1.39
Others and di- or tripeptides									
β-ala	8.70		7.07		7.91		6.99		0.85
Tau	138.49		116.62		152.38		151.25		15.08
Ans	28.24	a	17.53	c	20.94	bc	25.79	ab	1.95
Car	702.78		489.62		466.11		537.71		66.44
GSH	21.13	b	34.72	b	51.71	a	22.39	b	4.94
GSSG	223.89	a	121.65	b	124.19	b	82.31	c	9.36
Cys-cys	6.32		5.32		5.86		8.25		1.60

6 [a] Standard error of the mean.
7 [b] Different letters in the same row indicate significant differences at $p<0.05$ (Fisher's test).

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14 **Table 1. Pearson correlations between concentration of odor active volatile sulphur and nitrogen compounds and free amino acids throughout ripening**
15 **process.**

	Benzothiazole	**Pyrrole**	**2-Acetyl-1-pyrroline**	**2-acetyl-pyrrole**	**2,3-Dihydro-thiophene**	**2,6-Dimethyl-pyrazine**	**2-Ethyl-pyrazine**	**Methional**	**2-Ethyl-pyridine**
Asp	**0.960**	0.925	0.846	0.652	0.868	-0.927	0.762	**0.971**	0.819
Glu	0.860	0.867	0.770	0.278	0.678	-0.942	0.634	**0.963**	**0.910**
Ser	0.809	-0.992	0.068	0.418	0.035	**-0.998**	-0.114	0.702	**0.989**
Asn	0.743	**0.999**	**0.980**	0.349	0.942	**-0.999**	0.926	**0.931**	0.518
Gly	0.721	**1.000**	**0.998**	0.304	**0.965**	**-1.000**	**0.969**	**0.932**	0.524
Gln	0.826	0.990	0.940	0.334	0.883	-0.979	0.859	**0.989**	0.702
β-ala	-0.222	0.976	0.391	-0.427	0.357	0.986	0.533	0.137	-0.221
Tau	0.332	**0.999**	0.876	0.008	0.850	**0.998**	0.943	0.653	0.151
His	0.756	**0.998**	**0.980**	0.331	0.937	-0.993	0.924	**0.948**	0.557
Thr	0.701	**1.000**	**0.996**	0.319	**0.964**	**-1.000**	**0.962**	**0.910**	0.468
Ala	0.793	**1.000**	**0.978**	0.371	0.945	**-1.000**	0.924	**0.964**	0.601
Car	-0.436	0.909	0.256	-0.580	0.195	0.956	0.394	-0.054	-0.381
Arg	-0.516	-0.946	-0.822	0.243	-0.730	-0.782	-0.868	-0.759	-0.638
Pro	**0.929**	0.995	0.856	0.523	0.836	-0.995	0.754	**0.987**	0.793
Ans	-0.136	0.955	0.587	-0.392	0.521	0.943	0.694	0.276	-0.186
Tyr	**0.979**	0.345	0.503	0.712	0.530	-0.968	0.363	0.854	**0.896**
Val	0.855	**1.000**	0.949	0.423	0.918	**-0.999**	0.877	**0.988**	0.694
Met	**0.879**	**1.000**	0.932	0.419	0.895	**-1.000**	0.850	**0.999**	0.774
Ile	0.816	**1.000**	**0.973**	0.379	0.939	**-1.000**	0.914	**0.977**	0.645
Leu	**0.910**	**0.998**	0.889	0.492	0.864	**-0.997**	0.795	**0.992**	0.771
Phe	**0.891**	**0.997**	0.919	0.482	0.898	-0.993	0.837	**0.989**	0.726
Trp	**0.946**	0.989	0.827	0.552	0.811	-0.992	0.719	**0.983**	0.829
Orn	0.773	0.988	**0.966**	0.287	0.906	-0.957	0.899	**0.970**	0.627
Lys	0.720	0.997	**0.991**	0.287	0.945	-0.977	0.947	**0.936**	0.527
GSH	0.359	-0.929	-0.545	-0.103	-0.625	-0.892	-0.695	0.213	0.738
Cysteine	0.719	0.452	0.604	-0.036	0.442	-0.758	0.451	**0.884**	**0.879**
GSSG	-0.804	-0.813	-0.631	-0.661	-0.640	0.980	-0.498	-0.713	-0.527
Cystine	0.433	0.996	0.861	0.110	0.869	0.961	0.941	0.693	0.264

16 r values in bold indicate significant differences at $p<0.05$.

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